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The Di- and Tripeptide Transport Protein of *Lactococcus lactis*

A NEW TYPE OF BACTERIAL PEPTIDE TRANSPORTER*

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Lactococcus lactis **takes up di- and tripeptides via a proton motive force-dependent carrier protein. The gene (*dtpT*) encoding the di-tripeptide transport protein of *L. lactis* was cloned by complementation of a dipeptide transport-deficient and proline auxotrophic *Escherichia coli* strain. Functional expression of the dipeptide transport gene was demonstrated by uptake studies of alanyl-[^{14}C]glutamate and other peptides in *E. coli* cells. The di-tripeptide transport protein catalyzes proton motive force-driven peptide uptake and dipeptide exchange activity. The nucleotide sequence of *dtpT* was determined and the translated sequence corresponds with a protein of 463 amino acid residues. Hydropathy profiling indicates that the protein could form 12 membrane-spanning segments with the amino and carboxyl termini at the outer surface of the membrane. A secondary structure model is presented which is substantiated by analysis of DtpT-PhoA fusion constructs. Amino acid sequence comparisons showed no significant homology with other bacterial peptide transport systems nor with any other known protein. Flanking regions of the di-tripeptide transport gene were used to delete *dtpT* from the chromosome of *L. lactis*. Genetic and biochemical characterization of this mutant indicates that DtpT is the only transport protein in *L. lactis* for hydrophilic di- and tripeptides.**

Peptides can serve as sole carbon and/or nitrogen sources for most species of bacteria, fungi, plants, and animals (Payne, 1980). The best understood bacterial peptide transport systems are those from *Salmonella typhimurium* and *Escherichia coli*. These Gram-negative bacteria possess three distinct peptide transport systems with overlapping substrate specificities (Higgins and Gibson, 1986). The oligopeptide transport system (Opp) transports almost any peptide containing 2–5 amino acid residues (Hiles *et al.*, 1987). Apart from transporting nutrient peptides, Opp also serves an important function in the recycling of cell wall peptides which are released from peptidoglycan during growth (Goodell and Higgins, 1987). Furthermore, it plays a role in the sensitivity of enterobacteriaceae to aminoglycoside antibiotics (Kashiwagi *et al.*, 1992). The second peptide transport system (Tpp) has a more restricted substrate specificity. It only transports hydrophobic tripeptides and some dipeptides (Higgins and Gibson, 1986). This system is expressed only under anaerobic growth conditions. It is positively regulated by the gene products of *ompR* and *envZ* (Gibson *et al.*, 1987). The third peptide transport system is the dipeptide permease (Dpp). This transport system is rather specific for dipeptides but has also been shown to transport some tripeptides (Manson *et al.*, 1986). Dpp also serves as a chemoreceptor for peptide chemotaxis (Manson *et al.*, 1986).

For Gram-positive bacteria different peptide transport systems have also been described, *i.e.* the *ami* locus of *Streptococcus pneumoniae* (Alloing *et al.*, 1990), the Opp system of *Lactococcus lactis* (Kunji *et al.*, 1993) and the *dci* and *spoOk* loci of *Bacillus subtilis* (Mathiopoulos *et al.*, 1991; Perego *et al.*, 1991; Rudner *et al.*, 1991). All bacterial peptide transport systems described so far are members of a larger family, the ABC transporter or traffic ATPase superfamily. A typical ABC transporter consists of four membrane-associated domains (Higgins, 1992). Two of these domains are highly hydrophobic and span the membrane (normally) six times. These domains form the pathway through which the substrate crosses the membrane. The two other domains contain the ATP-binding cassette and are located at the cytoplasmic face of the membrane. All bacterial ABC transporters that mediate solute uptake require a substrate-binding protein. Binding proteins of Gram-negative bacteria are located in the periplasm. The substrate-binding proteins of the Gram-positive bacteria have a signal peptide with a sequence typical for lipomodification (Perego *et al.*, 1991).

The di-tripeptide carrier of *L. lactis* (DtpT) is a secondary transport system in contrast to the binding protein-dependent ATP-driven peptide transport systems. Accumulation of the dipeptide alanylglutamate in peptidase-free membrane vesicles of *L. lactis* has been shown to be driven by the electrical potential ($\Delta\Psi$) and the chemical gradient of protons (ΔpH) across the membrane (Smid *et al.*, 1989a). The DtpT system has a broad substrate specificity, but size recognition is restricted to di- and tripeptides only.

In this study, the gene encoding the lactococcal di-tripeptide transport system was cloned and characterized genetically and biochemically. The high specificity for proline-containing dipeptides was used to complement the proline auxotrophic, dipeptide transport negative *E. coli* E1772. The cloned gene encodes an integral membrane protein with a unique primary sequence and some unusual secondary structure features.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. *E. coli* strains were grown at 37 °C, with vigorous aeration, in Luria Broth or in M9 minimal medium (Sambrook *et al.*, 1989) supplemented with carbenicillin (50 μg/ml), tetracycline (10 μg/ml), or kanamycin sulfate (50 μg/ml) when appropriate and essential nutrients as indicated by the auxotrophic markers. *L. lactis* strains were grown at 28 °C in M17 (Difco) or in a chemically defined medium (Poolman and Konings, 1988), both at pH 6.4, and supplemented with 0.5% (w/v) glucose.

Cloning of the Transport Gene—Chromosomal DNA was isolated from *L. lactis* ML3 according to Leenhouts *et al.* (1990) and partially digested with endonuclease *Sau*3A. The cleaved DNA was fractionated by polyacrylamide gel (5% w/v) electrophoresis, after which fragments

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TABLE I
Bacterial strains and plasmids

Bacterium/plasmid	Relevant characteristics	Source/refs.
Bacterium		
E. coli		
E1772	W3110, *dppA*20::Kan, *proC*::Tn10Δ*lacU169*	Olson *et al.* (1991)
P678-54	*lacY1 min A1 min B2*	Adler *et al.* (1967)
BX2	LE392, *pcn*5zad::Tek	François *et al.* (1987)
CC118	Δ(*ara,leu*)7697 Δ*lacX*74 *phoA*Δ20 *galE galK thi rpsE rpoB argE*(am) recA1	Manoil (1990)
JM101	*supE thi* Δ*(lac-proAB)* F'[*traD36proAB*$^+$*lacI*^{q}*lacZ M15*]	Sambrook *et al.* (1989)
L. lactis		
ML3	Wild-type	
MG1363	ML3, plasmid free, Lac$^-$, Prt$^-$	Gasson (1983)
MGDT1	MG1363, DtpT$^-$	Kunji *et al.* (1993)
MG1363-3	MG1363, pINT300 integrated in chromosome	This work
AG300	MG1363, Δ*dtpT*	This work
Plasmid		
pTAQI	pBR322, *lacI* behind penicillinase promoter, expression vector with *tac* promoter	Genencor Int., South San Francisco
pDT5	pTAQI, carrying *dtpT* gene of *L. lactis* on a 4.2-kb fragment	This work
pBluescript II SK$^+$	AP^R, expression vector	Stratagene
pSKF3	pBluescript II SK$^+$, carrying *dtpT* of *L. lactis* on 3.5-kb *Hin*dIII-*Sal*I fragment	This work
pJF751	Ap^R, *phoA* lacking signal sequence	Genentech, South San Francisco
pPHO7	Ap^R, *lacZ* lacking translation initiation signals	Gutierrez and Devedjian (1989)
pWV01	cryptic plasmid from *L. lactis* subsp. *cremoris* WG2	Vosman and Venema (1983)
pORI28	Em^R, LacZ$^+$, deletion derivative of pWV01 lacking *repA*	Leenhouts and Venema (1993)
pINT300	pORI280, containing flanking regions of *dtpT* of *L. lactis*	This work
pGKV210	Em^R, *E. coli-L. lactis* shuttle vector	Van der Vossen *et al.* (1985)
pGKF5	pGKV210, containing *dtpT* of *L. lactis on 4.2-kb fragment*	This work

1–15 kilobases (kb)[1] in length were electroeluted from the gel. The chromosomal DNA fragments were mixed with the expression vector pTAQI that had been linearized with restriction enzym *Bam*HI and dephosphorylated. After ligation, the resulting chimeric plasmids were used to transform electrocompetent *E. coli* cells (Dower *et al.*, 1988). The transformed organisms were spread on M9 plates, supplemented with carbenicillin (50 μg/ml), 100 μM isopropyl 1-thio-β-D-galactopyranoside, and 100 μM prolylglycine (Pro-Gly) as sole proline source.

DNA-DNA Hybridization—Chromosomal DNA was digested with the appropriate restriction enzyme(s) and fractionated by agarose gel electrophoresis. DNA was transferred to GeneScreen Plus filters (DuPont-New England Nuclear) by using the protocol of Southern, modified by Chomczynski and Qasba (1984). DNA was labeled with digoxigenin-dUTP by using Nonradioactive DNA Labeling and Detection Kit (Boehringer). Hybridization, washing, and staining steps were performed according to instructions of the manufacturer.

Transport Assays—Cells grown to an OD_{660} of 0.6 were harvested by centrifugation, washed two times, and resuspended in 100 mM potassium phosphate, pH 6.5, to a final OD_{660} of approximately 25. The cells were de-energized with 10 mM 2-deoxy-glucose for 20 min at 28 °C. This procedure results in the depletion of intracellular amino acid pools (Poolman *et al.*, 1987). De-energized cells were washed twice and resuspended as indicated in the legends to figures. All transport assays were performed at 28 °C. Uptake of radioactively labeled compounds in intact cells was studied as previously described by Smid *et al.* (1989b). Uptake of unlabeled peptides was monitored by determining the intracellular concentration of the corresponding amino acids by means of reversed-phase high performance liquid chromatography analysis as has been described previously (Kunji *et al.*, 1993).

Identification of DtpT in Minicells—Minicell-producing *E. coli* P678–54 was transformed with pDT5 (expression of DtpT) and pTAQI (vector control), and cells were grown in M9 minimal medium supplemented with 2% (w/v) cas-amino acids. Minicells were isolated and purified in three subsequent sucrose gradient centrifugations (Meager *et al.*, 1977). *In vivo* labeled ([^{35}S]methionine, 1,100 Ci/mmol, Amersham Corp.) proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The ^{35}S-labeled proteins were identified by autoradiography.

Molecular Cloning and DNA Sequencing—Molecular cloning techniques were performed essentially as described by Sambrook *et al.* (1989). Plasmids from *L. lactis* were isolated by the method of Birnboim and Doly with modifications described by Leenhouts *et al.* (1990). *L. lactis* was transformed by electroporation as described by Holo and Nes (1989). For DNA sequencing, the 3.5-kb *Hin*dIII-*Sal*I insert of pDT5 (see Fig. 2) was transferred to pBluescript II SK$^+$ (Stratagene). To facilitate cloning of this fragment, and fragments derived thereof, *E. coli* BX2, which reduces the copy number of Col E1 ori plasmids, was used as host. Subclones of pSKF3 (3.5-kb *Hin*dIII-*Sal*I insert of pDT5 in pBluescript II SK$^+$) were also obtained by exonuclease digestion using the Erase-a-base method (Promega). A T7 DNA sequencing kit (Pharmacia) was used for sequencing of double-stranded DNA by the dideoxy chain termination method (Sanger *et al.*, 1977). PCGENE (release 6.26, Genofit) was used for computer-assisted sequence analysis. Protein homology searches were performed in the EMBL SWISSPROT (release 23) data base, using the FASTA algorithm from Pearson and Lipman (1988).

Construction of the Integration Plasmid pINT300—The integration plasmid pINT300, which contains the 5'- and 3'-flanking sequences of *dtpT* was constructed as follows. *Sal*I-digested pSKF3 was treated with Klenow enzyme to fill in the recessed ends resulting in the deletion of the *Sal*I site. After ligation, this plasmid was cut with *Hin*cII to obtain a 5.6-kb fragment (pBluescript containing the 5'- and 3'-flanking sequences of *dtpT*) and a 1-kb fragment (most of the *dtpT* gene). The 5.6-kb fragment was digested with *Bam*HI to facilitate transfer of the 5'- and 3'-flanking sequences (chromosomal fragments A and B, see Fig. 6) to pORI280 (Leenhouts *et al.*, 1992). The corresponding plasmid was named pINT300.

Protein Determination—Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Translational Fusions—Plasmid pJF751 was used for constructing fusions of *dtpT* and the β-galactosidase gene (*lacZ*). pJF751 is devoid of transcription and translation initiation signals, and, consequently, does not express β-galactosidase activity. The 5' region of *dtpT* was synthesized by the polymerase chain reaction (PCR) using appropriate oligonucleotide primers in which a *Bam*HI site was present (forward primer, 5'-GTTTGTTATGGATCCTCTTTTTC; reverse primer, 5'-TTGTCAATGGATCCAGGTAATA). Following digestion with *Bam*HI, the PCR fragments were inserted in frame with the *lacZ* gene of pJF751. The resulting chimeric plasmids containing fusions with *lacZ* were transformed to *E. coli* JM101. These transformants were plated on LB with 50 μg/ml carbenicillin and 40 μg/ml X-gal, and analyzed for expression of β-galactosidase activity (Miller, 1972).

[1] The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ORF open reading frame; bp, base pair(s); diACA, alanyl-β-chloro-alanine.

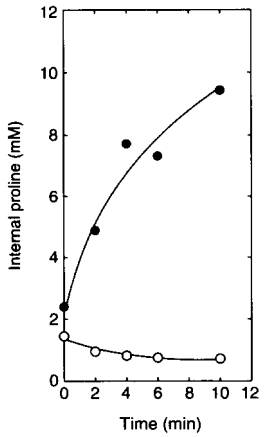

FIG. 1. **Transport of prolylglycine in *L. lactis* ML3 wild-type (●) and di- and tripeptide transport deficient mutant MGDT1(○).** Concentrated cell suspensions were diluted to a final protein concentration of about 0.3 mg/ml into 100 mM potassium-MES, 5 mM $MgSO_4$, pH 6.5. After 4 min of pre-energization with 25 mM glucose, Pro-Gly was added to a final concentration of 1 mM. Transport was stopped at different time intervals and the samples were analyzed by reversed-phase HPLC as described under "Materials and Methods."

For constructing fusions of *dtpT* and the alkaline phosphatase gene (*phoA*), plasmid pPH07 was used. pPH07 contains *phoA*, which lacks promoter and leader sequence. The second multiple cloning site of pPH07 was removed as a *Xho*I-*Sac*I fragment, yielding pPH07A. The 5′ region of *dtpT*, and various portions of the coding regions, were synthesized by PCR using appropriate oligonucleotide primers in which *Bam*HI or *Sma*I sites were present (for details, see legend to Fig. 9). Following digestion with *Bam*HI and *Sma*I, the PCR fragments were inserted in frame with the *phoA* gene of pPH07A. The resulting chimeric plasmids containing fusions with the *phoA* gene were transformed to *E. coli* CC118. The transformants were plated on LB with 50 μg/ml carbenicillin and 40 μg/ml 5-bromo-3-chloro-indolyl-phosphate-P-toluidine and analyzed for expression of alkaline phosphatase activity (Michaelis *et al.*, 1983).

The expression of DtpT/PhoA fusion proteins was detected by immunoblotting with an antibody directed against alkaline phosphatase. *E. coli* CC118 cells carrying the the constructed plasmids were used to estimate the expression of the fusion proteins. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to polyvinylidiene difluoride membranes by semi-dry electrophoretic blotting. Processing of the polyvinylidiene difluoride membranes (antibody binding, serum dilution of 10,000) and immunoblot detection using a secondary alkaline phosphatase-labeled anti-rabbit IgG was performed as described (Harlow and Lane, 1988). Each of the fusion proteins was detected but the expression levels varied somewhat (data not shown).

Chemicals—Alanyl-[^{14}C]glutamate (57 mCi/mmol) was synthesized as described previously (Smid *et al.*, 1989a). All other peptides were obtained from Sigma or Bachem Feinchemikalien AG, Bubendorf, Switzerland. All peptides and amino acids used were in the L-configuration. All chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Cloning of the Di-tripeptide Transport Gene—The di-tripeptide transport gene of *L. lactis* ML3 was cloned by complementation of *E. coli* E1772, which is proline auxotroph and dipeptide transport negative. Although *E. coli* E1772 is Dpp$^-$, it still transports various proline-containing dipeptides (*e.g.* prolylleucine and prolylmethionine) most likely as a result of Opp and/or Tpp activity. However, the strain is unable to take up prolylglycine (Pro-Gly) and this property was used in our complementation assay. To ascertain that Pro-Gly is a substrate of the di-tripeptide carrier of *L. lactis*, uptake of Pro-Gly was assayed in washed cell suspensions and accumulation was quantitated from the increase in intracellular proline as measured by reversed-phase HPLC. The results presented in Fig. 1 show that Pro-Gly is taken up by the wild-type ML3 strain, while uptake is not observed with the di-tripeptide transport negative mutant *L. lactis* MGDT1 (Kunji *et al.*, 1993).

For shotgun cloning, fractionated *L. lactis* chromosomal DNA was ligated in the expression vector pTAQI. pTAQI was used as the cloning vector since DNA fragments could be inserted downstream of the *tac* promoter. This allows expression of proteins in case the cloned genes do not possess a promoter or in case the *L. lactis* promoter is not functional in *E. coli*. The ligation mixtures were used to transform *E. coli* E1772, after which the cells were spread on M9 plates containing carbenicillin and Pro-Gly as sole source of proline. Colonies can only grow on these selective plates when *E. coli* E1772 is functionally complemented by the di-tripeptide transport gene of *L. lactis*. Five colonies were found on the selective plates after transformation. These colonies did not grow on plates without a proline source, which excludes the possibility that genes coding for proline biosynthesis functions were cloned. Analysis of plasmid DNA of one of the colonies showed that the pTAQI vector contained a 4.2-kb insert. This chimeric plasmid was designated pDT5.

To confirm that the gene for di-tripeptide transport originated from the *L. lactis* chromosome, chromosomal DNA of *L. lactis* was digested with *Hin*dIII and *Kpn*I and fractionated by agarose electrophoresis. A digoxigenin-11-dUTP-labeled 2.1-kb *Hin*dIII-*Kpn*I fragment of the 4.2-kb insert of pDT5 (see Fig. 2) hybridized with a 2.1 chromosomal fragment. This probe did not hybridize to chromosomal DNA of *E. coli* E1772 (data not shown).

Nucleotide Sequence and Coding Regions of the Di- and Tripeptide Transport Gene—A restriction map of the 4.2-kb insert was constructed by double and triple digestions. The map is shown together with the flanking regions of the pTAQI vector in Fig. 2. Restriction sites for the enzymes *Sal*I, *Sac*I, *Eco*RI, *Sph*I, and *Sma*I were not present in the 4.2-kb fragment. The restriction map was used to locate the di-tripeptide transport gene on pDT5 by subcloning fragments. Maps of the plasmid derivatives and the growth characteristics of *E. coli* E1772 transformed with these plasmids on selective plates with prolylglycine are shown in Fig. 2*B*. Only *E. coli* E1772 bearing plasmid pDT3 was able to grow on the selective plates. The 3.5-kb *Hin*dIII-*Sal*I fragment was transferred to pBluescript II SK$^+$, yielding pSKF3. Subclones of pSKF3 were obtained by exonuclease digestion, and the nucleotide sequence was determined.

The nucleotide sequence of the di-tripeptide transport gene and the 5′- and 3′-flanking regions is shown in Fig. 3. Between positions 283 and 1672 an open reading frame (ORF) of 1,389 bp is found. This ORF could encode a protein (DtpT) of 463 amino acids, corresponding with a molecular mass of 50,630 daltons. The ORF is preceded by a putative ribosome-binding site, GGAG at position 261 (Fig. 3). This ribosome-binding site is complementary to the 3′ end of the lactococcal 16 S rRNA (Ludwig *et al.*, 1985) and has a ΔG of −9.4 kcal (Tinoco *et al.*, 1973). If translation occurs at position 283, the spacing between the ribosome-binding site and the ATG is 18 bases which is rather long for efficient translation of the mRNA. Another potential translation initiation site is the ATG at position 364, with a putative ribosome-binding site AAAG (ΔG is −4.6 kcal) at bp 352. To establish whether the first ribosome-binding site and the ATG at position 283 could serve as start signal in *E. coli*, the 5′ region of the putative ORF and part of the coding region (bp 78–300) were fused to *lacZ* lacking translation initiation signals. The corresponding construct (pDTZ1) yielded high β-galactosidase activity (425 Miller units) when present in *E. coli* JM101 (control cells without pDTZ1 had a β-galactosidase activity of 5 Miller units). These results suggest that translation initiation of the gene in *E. coli* does occur at the position

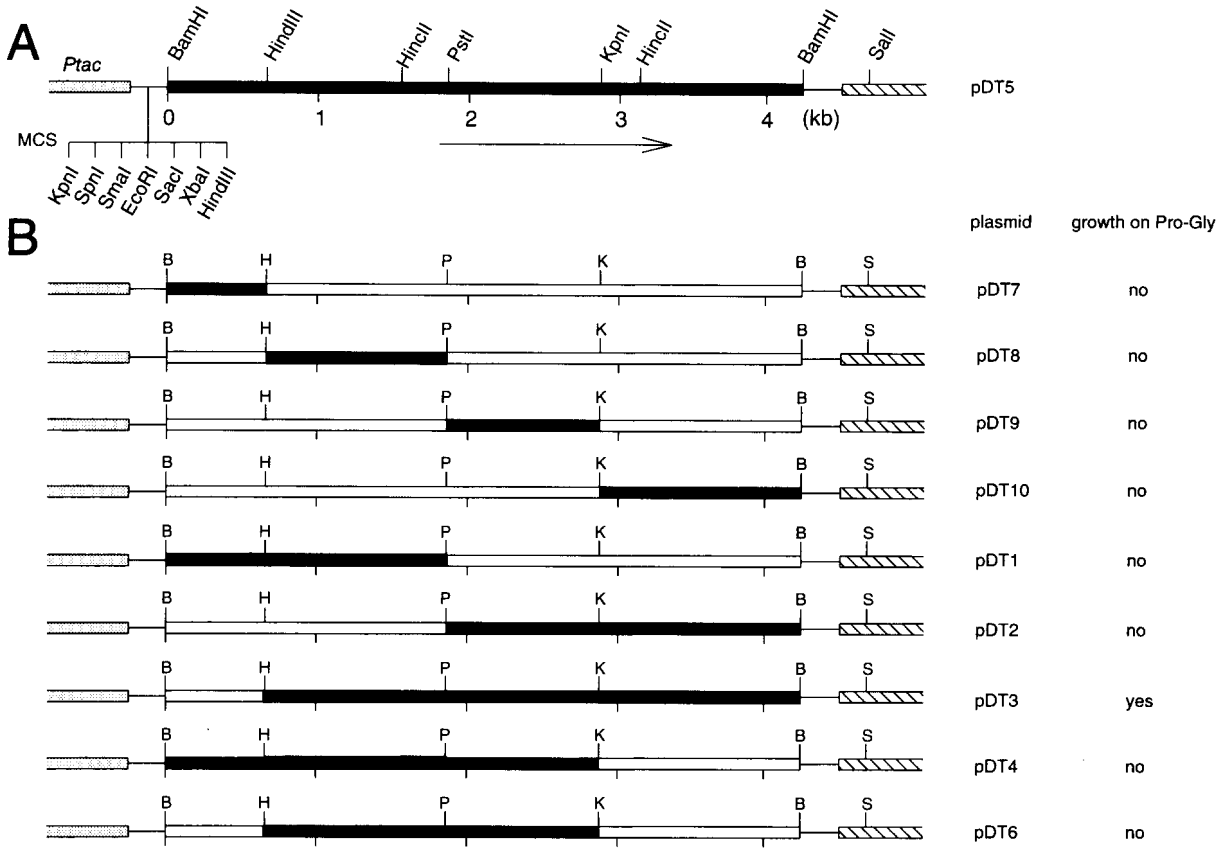

FIG. 2. **Restriction endonuclease map of pDT5 (*A*) and characterization of the deletion derivatives of pDT5 (*B*).** *E. coli* E1772 cells transformed with these plasmids were analyzed for growth on selective media with prolylglycine as sole source of proline. P_{tac} and *MCS* refer to the *tac* promoter and the multiple cloning site of pTAQI, respectively. Symbols: *open bars*, deletions in the cloned fragment; *dotted bars* and *striped bars*, P_{tac} and vector DNA, respectively. The position and the direction of transcription of the di-tripeptide transport gene are indicated by the *arrow*. *H*, *Hin*dIII; *P*, *Pst*I; *K*, *Kpn*I; *B*, *Bam*HI; *S*, *Sal*I.

283 ATG. The analysis of the secondary structure of the protein (see below, Fig. 9) indicates the presence of a transmembrane protein segment in the coding region of bp 285–350, thereby providing some evidence that the same start site may be used in *L. lactis*.

Upstream of the di- and tripeptide transport gene strong consensus promoter sequences are found, *i.e.* TTGACA (-35) at position 144, TATAAT (-10) at position 167 with a spacing of 17 bp. Clearly, these promoter elements are in accordance with those recognized by RNA polymerases from both Gram-negative and Gram-positive bacteria. Downstream of the stop codon of the ORF (at position 1672), an inverted repeat is found (ΔG is -16.2 kcal), which could form a rho-independent terminator-like structure. The GC content of *dtpT* is 38%, which is common for lactococcal genes. The codon usage is in agreement with that tabulated for other sequenced genes of *L. lactis* (van de Guchte *et al.*, 1992). DtpT contains 69.1% non-polar amino acid residues, indicating a composition typical for membrane proteins.

Identification of the Di-tripeptide Transport Protein in Minicells—In the minicell-producing strain *E. coli* P678–54, in which pDT5 was used to express the di-tripeptide transport protein, one additional protein band with an apparent molecular mass of 43 kDa was found which is not present in a control strain bearing pTAQI (Fig. 4). The discrepancy between the molecular mass (50,630 Da) deduced from the nucleotide sequence and the apparent molecular mass determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is probably due to the abnormally high binding of sodium dodecyl sulfate to the di- and tripeptide transport system, similar to that observed for other hydrophobic proteins (Poolman *et al.*, 1989; Tolner *et al.*, 1992). The concentration of β-lactamase and lac repressor protein (LacI) in minicells harboring pDT5 is much lower than in minicells containing pTAQI, suggesting that the insert reduces the copy number of the plasmid.

Functional Properties of the Di-tripeptide Transport Protein—Alanylglutamate (Ala-Glu) has previously been used to characterize the *L. lactis* di-tripeptide transport protein in membrane vesicles (Smid *et al.*, 1989a). For this purpose L-alanyl-[^{14}C]glutamate was synthesized. Ala-Glu transport activity was assayed in the dipeptide transport deficient *E. coli* E1772 to demonstrate that the di-tripeptide transport gene of *L. lactis* is functionally expressed. Uptake of Ala-[^{14}C]Glu by E1772 harboring pDT5, which carries the cloned di-tripeptide transport gene, is severalfold higher than in cells harboring pTAQI (Fig. 5*A*). To establish that Pro-Gly (used in the complementation assay) and Ala-Glu are taken up via the same transport system, competition experiments were performed. The initial rate of uptake and steady-state level of accumulation of the radioactive Ala-Glu decreased significantly upon addition of a 10-fold excess of unlabeled Pro-Gly to the reaction mixture (Fig. 5*B*). Complete inhibition of Ala-[^{14}C]Glu uptake was observed with a 100-fold excess of Pro-Gly, indicating that Pro-Gly and Ala-Glu indeed compete for the same transport system. To assure that Ala-[^{14}C]Glu was the transported species rather than [^{14}C]glutamate, which could have been formed from extracellular or periplasmic peptidase activity, unlabeled glutamate was added to the reaction mixture. Addition of glutamate had no effect on the uptake of labeled Ala-Glu (data not shown), indicating that hydrolysis of the dipeptide had not occurred prior to transport. These results confirm the specificity of the system for dipeptides. The specificity for peptides is also shown by the

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AGTTCTCACGGAAATTTATTATTTATGAGAAAATTAAAGACTTATCTTAGAAATGTTTTTA   60

AAAGAAATACCTTGAAAAGTTTGTTATTGTCCCTCTTTTTCCGCGTTTTTCAAAGAATCT  120
                -35          -10
GTATAGGCAAAATTGTTTGACAACTTGACAAGTTGTATAGAAAACTTTATAATATAATCT  180
ATGCAAAACTTAAACAAAACAGAAAAAACATTTTTCGGTCAACCTCGCGGCCTATTGACG  240
CTTTTTCAGACTGAGTTCTGGGAGCGTTTTTCTTACTATGGGATGCGTGCCATTCTGGTT  300
                                        M  R  A  I  L  V

TATTACCTCTATGCATTGACAACTGCAGATAACGCAGGTTTAGGACTTCCTAAAGCTCAG  360
Y  Y  L  Y  A  L  T  T  A  D  N  A  G  L  G  L  P  K  A  Q

GCAATGGCGATTGTAAGTATTTATGGTGCACTTGTCTATCTTTCAACAATTGTTGGGGGA  420
A  M  A  I  V  S  I  Y  G  A  L  V  Y  L  S  T  I  V  G  G

TGGGTTGCTGACCGGTTGTTGGGCGCTTCGCGCACAATCTTCTTGGGTGGTATTTTAATC  480
W  V  A  D  R  L  L  G  A  S  R  T  I  F  L  G  G  I  L  I

ACTTTAGGACACGTCGCTTTAGCAACACCATTTGGTTTATCTTCACTCTTCGTGGCATTA  540
T  L  G  H  V  A  L  A  T  P  F  G  L  S  S  L  F  V  A  L

TTCTTGATTATCTTAGGAACAGGGATGCTTAAACCCAATATTTCTAACATGGTTGGGCAT  600
F  L  I  I  L  G  T  G  M  L  K  P  N  I  S  N  M  V  G  H

CTATATTCAAAAGATGACTCACGTCGTGATACTGGATTTAATATCTTTGTAGTCGGAATT  660
L  Y  S  K  D  D  S  R  R  D  T  G  F  N  I  F  V  V  G  I

AATATGGGTTCTCTGATTGCTCCATTGATTGTTGGGACAGTTGGACAAGGCGTGAACTAC  720
N  M  G  S  L  I  A  P  L  I  V  G  T  V  G  Q  G  V  N  Y

CACTTAGGTTTCTCACTTGCCGCAATCGGAATGATTTTTGCATTATTTGCTTATTGGTAT  780
H  L  G  F  S  L  A  A  I  G  M  I  F  A  L  F  A  Y  W  Y

GGACGTCTTCGTCATTTCCCAGAAATTGGACGTGAACCATCTAATCCAATGGATGCAAAA  840
G  R  L  R  H  F  P  E  I  G  R  E  P  S  N  P  M  D  A  K

GCAAAACGTAATTTTATTATTACATTAACGATTGTTCTTATCGTTGCTTTAATCGGATTT  900
A  K  R  N  F  I  I  T  L  T  I  V  L  I  V  A  L  I  G  F

TTCTTAATTTATCAAGCAAGTCCTGCGAATTTCATCAATAATTTCATTAACGTTTTATCA  960
F  L  I  Y  Q  A  S  P  A  N  F  I  N  N  F  I  N  V  L  S

ATTATCGGTATTGTTGTTCCAATTATTTATTTCGTAATGATGTTTACCTCTAAAAAGGTA 1020
I  I  G  I  V  V  P  I  I  Y  F  V  M  M  F  T  S  K  K  V

GAATCAGACGAACGTCGTAAATTAACGGCTTATATTCCTTTGTTCCTTTCTGCTATTGTC 1080
E  S  D  E  R  R  K  L  T  A  Y  I  P  L  F  L  S  A  I  V

TTTTGGGCAATTGAAGAACAAAGTTCTACGATTATTGCGGTTTGGGGAGAATCACGTTCT 1140
F  W  A  I  E  E  Q  S  S  T  I  I  A  V  W  G  E  S  R  S

AACTTAAATCCTACTTGGTTTGGATTTACTTTCCATATTGACCCATCTTGGTACCAATTG 1200
N  L  N  P  T  W  F  G  F  T  F  H  I  D  P  S  W  Y  Q  L

TTGAACCCACTCTTCATCGTTCTCTTGTCACCTATCTTTGTACGAATTTGGAACAAATTA 1260
L  N  P  L  F  I  V  L  L  S  P  I  F  V  R  I  W  N  K  L

GGAGATCGTCAACCATCAACCATCGTTAAATTTGGTCTTGGACTGATGTTGACCGGAGCT 1320
G  D  R  Q  P  S  T  I  V  K  F  G  L  G  L  M  L  T  G  A

TCTTATTTGATTATGACACTTCCTGGACTCTTGAATGGGACTTCTGGACGTGCGAGTGCT 1380
S  Y  L  I  M  T  L  P  G  L  L  N  G  T  S  G  R  A  S  A

CTTTGGCTAGTATTGATGTTTGCTGTTCAAATGGCAGGTGAATTACTTGTTTCACCAGTT 1440
L  W  L  V  L  M  F  A  V  Q  M  A  G  E  L  L  V  S  P  V

GGTTTATCAGTTTCAACAAAATTAGCGCCAGTAGCATTCCAATCTCAAATGATGGCAATG 1500
G  L  S  V  S  T  K  L  A  P  V  A  F  Q  S  Q  M  M  A  M

TGGTTCTTGGCAGACTCAACTTCACAAGCGATTAATGCCCAAATTACACCTATCTTTAAA 1560
W  F  L  A  D  S  T  S  Q  A  I  N  A  Q  I  T  P  I  F  K

GCAGCAACAGAAGTTCACTTCTTTGCAATTACAGGGATTATCGGTATTATCGTTGGAATC 1620
A  A  T  E  V  H  F  F  A  I  T  G  I  I  G  I  I  V  G  I
                                                  stop
ATCCTCCTTATTATCAAAAAACCTATTTTGAAATTAATGGGAGATGTTCGTTAATTAGTA 1680
I  L  L  I  I  K  K  P  I  L  K  L  M  G  D  V  R  -

AAGGAATGAAAAAACCTATCTTGAGAGATAGGTTTTTTTGTATCTTTAAAATGACATTGT 1740
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FIG. 3. **Nucleotide sequence of the di- tripeptide transport gene and the flanking regions.** The start and stop codons, putative promoter (–35/–10), possible ribosome-binding site and possible terminator sequences (→←) are indicated. The amino acid sequence deduced from the DNA sequence is shown below the DNA sequence. The 12 transmembrane α-helical segments as predicted by the hydropathy profiling method of Eisenberg *et al.* (1984) are *underlined*.

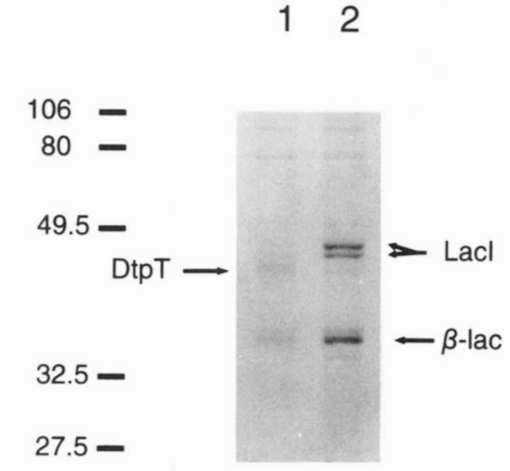

FIG. 4. **Expression of DtpT in minicell-producing *E. coli* P678–54.** Proteins were labeled in the presence of [^{35}S]methionine and resolved by 12.5% SDS-polyacrylamide gel electrophoresis. *Lane 1*, P678–54 containing pDT5; *lane 2*, P678–54 containing pTAQI (vector control). Di-tripeptide transport protein (*DtpT*), lac repressor protein (*LacI*), β-lactamase (*β-lac*), and molecular size markers (*kDa*) are indicated.

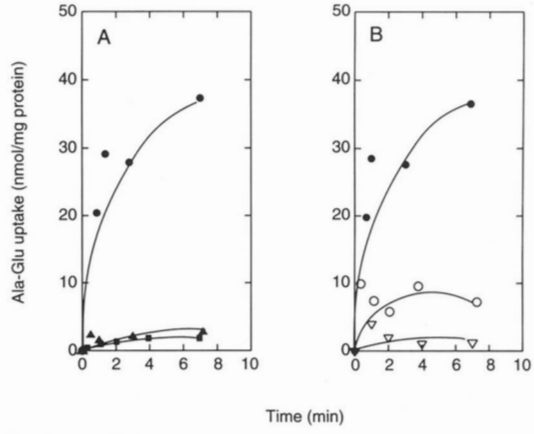

FIG. 5. **Alanyl-[^{14}C]glutamate uptake in *E. coli* E1772 cells harboring pTAQI (▲) or pDT5 (▽, ●, ○, ■).** Concentrated cell suspensions were diluted to a final protein concentration of ~0.33 mg/ml into 50 mM potassium phosphate, 5 mM $MgSO_4$, pH 6.5. After 1 min of pre-energization with 10 mM D-lactate in the presence of oxygen, Ala-[^{14}C]Glu was added to a final concentration of 478 μM. *Closed squares* show the uptake of Ala-[^{14}C]Glu in the presence of 55 μM CCCP. Transport was stopped at different time intervals as described under "Materials and Methods." *B*, *open symbols* indicate the uptake of Ala-[^{14}C]Glu in the presence of a 10-fold (○) and 100-fold (▽) excess of unlabeled Pro-Gly.

exit of accumulated Ala-[^{14}C]Glu in *E. coli* strain E1772 harboring pDT5 upon addition of a 10-fold excess of unlabeled Ala-Glu. Exit of Ala-[^{14}C]Glu was not observed upon addition of unlabeled glutamate (Fig. 6). Reversed-phase HPLC analysis of a cytoplasmic extract of *E. coli* E1772/pDT5 showed that *E. coli* is unable to hydrolyze Ala-Glu unlike most other peptides (data not shown), which is consistent with the observed Ala-[^{14}C]Glu/Ala-Glu exchange (Fig. 6). Evidence for a role of the Δp as the driving force for dipeptide transport comes from studies with the protonophore CCCP. Results presented in Fig. 5*A* show that CCCP inhibits the uptake of Ala-Glu by the DtpT transport protein expressed in *E. coli*. From these results, we conclude tentatively that the cloned *dtpT* gene encodes the H^+-linked peptide transport system that was previously identified in *L. lactis* (Smid *et al.*, 1989).

Construction and Analysis of a Di-tripeptide Transport-negative Mutant of L. lactis—To prove that DtpT is the lactococcal di-tripeptide transport protein, and to obtain a stable dipeptide transport-negative mutant suitable for expression of various *dtpT* alleles in *L. lactis*, the *dtpT* gene was deleted from the chromosome via homologous recombination (Fig. 7*A*). For this purpose plasmid pINT300 was constructed, which is a derivative of pORI280 (Leenhouts and Venema, 1993). Plasmid pORI280 contains a functional β-galactosidase gene (*lacZ*), an erythromycin-resistant gene (Em^R) and a multiple cloning site. The plasmid lacks the *repA* gene and can only replicate in a host strain which provides the *repA* gene in *trans* (Leenhouts *et al.*, 1991). Flanking regions of the *dtpT* gene, indicated by *A* and *B* in Fig. 7*A*, were inserted in the multiple cloning site of

pORI280 yielding pINT300. *L. lactis* MG1363 was transformed with pINT300 and transformants were selected on M17, supplemented with X-gal and erythromycin. Blue colonies arise from cells in which recombination between the chromosome and one of the flanking regions has occurred. Southern hybridization analysis of chromosomal DNA isolated from several transformants demonstrated that the plasmid had integrated into the genome at each of the homologous loci (data not shown). Fig. 7*B* (*lane 2*) shows a 2.6- and a 5.2-kb fragment, as expected upon integration in region A (strain MG1363–3). Subsequently, *L. lactis* MG1363–3 was grown for 30 generations in nonselective media, *i.e.* without erythromycin. This allows the plasmid to resolve at the other homologous region (*B*), which leads to deletion of the transport gene from the chromosome. *L. lactis* lacking *dtpT* was selected on M17 X-gal plates. A number of white colonies were selected and further analyzed by Southern hybridization. The results of the analysis of one of these colonies is presented in Fig. 7*B* (*lane 3*). The detection of a 4.2-kb chromosomal fragment indicates that strain MG1363–3 has lost all vector sequences, but also most (1088 bp) of the *dtpT* gene. This mutant was designated *L. lactis* AG300.

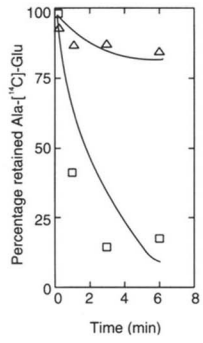

FIG. 6. **The effect of an excess of unlabeled glutamate (△) or Ala-Glu (□) (5 mM, final concentration) on Ala-[^{14}C]Glu exit in E1772/pDT5 cells, which had accumulated Ala-[^{14}C]Glu for 4 min as described under Fig. 5 (●).**

To characterize the *dtpT* deletion strain, growth experiments were performed first. *L. lactis* AG300 grew normally on chemically defined medium containing 0.25 mM of the dipeptide analog alanyl-β-chloro-alanine (diACA), which is toxic for the wild-type *L. lactis*. DiACA has previously been shown to be a substrate of the di-tripeptide transport system of *L. lactis* (Smid *et al.*, 1989b). On the other hand, the mutant was not able to grow on chemically defined medium containing 1 mM alanylglutamate as sole source of glutamate. Glutamate is an essential amino acid for *L. lactis* (Smid, 1991). These growth experiments indicate that the di- and tripeptide transport system is not functional in the deletion mutant AG300. Second, uptake of prolylalanine (Pro-Ala) was monitored in MG1363 (wild-type) and AG300 (Δ*dtpT*). Transport of Pro-Ala is completely abolished in the mutant, while initial uptake rates of 15 nmol/min·mg of protein could be estimated in the parent (wild-type) strain (Fig. 8). Finally, *L. lactis* AG300 was transformed with plasmid pGKF5, which contains the di-tripeptide transport gene. *L. lactis* AG300/pGKF5 had the same growth characteristics (sensitivity to diACA, growth on Ala-Glu) as the wild-type. These results demonstrate that the *dtpT* gene has been deleted from the chromosome of *L. lactis* MG1363, result-

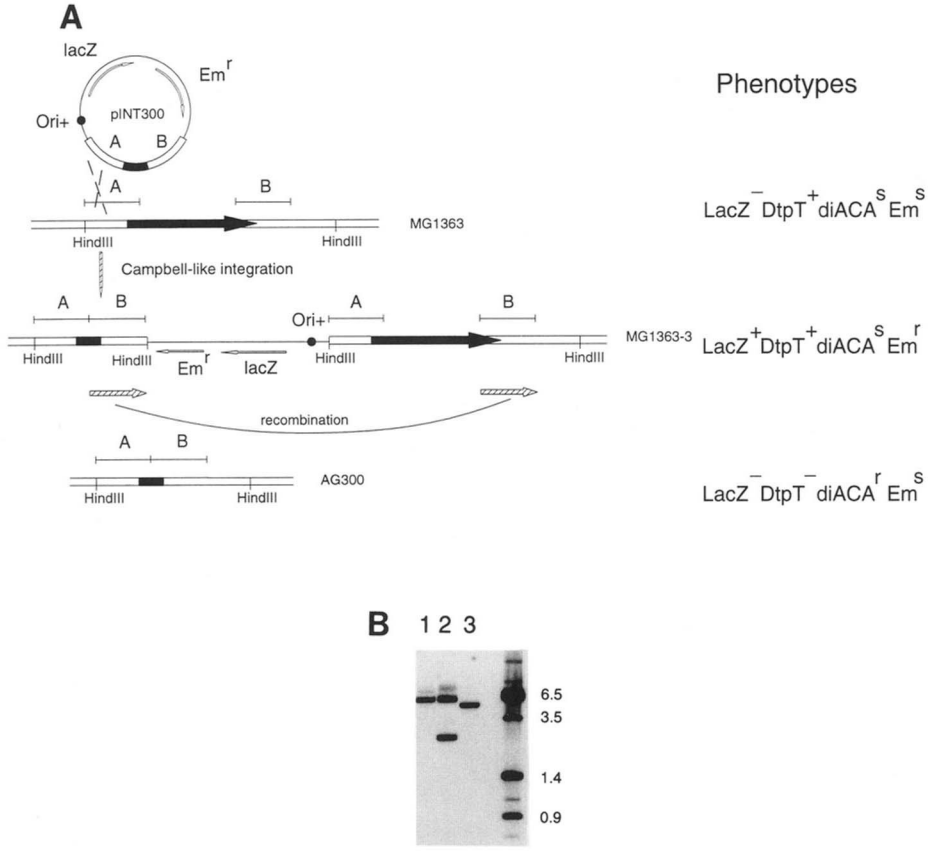

FIG. 7. *A*, schematic representation of the strategy resulting in deletion of the *dtpT* gene from the *L. lactis* MG1363 chromosome. *Arrow*, *dtpT* (di- and tripeptide transport gene); *line*, vector sequence including Em^R (erythromycin resistance gene), *lacZ* (β-galactosidase gene) expressed under control of the lactococcal promoter P_{32}, and ori$^+$ (origin of replication of the lactococcal plasmid pWV01); *open bar*, *L. lactis* DNA; *A* and *B*, the flanking regions and a small part of the 5′ and 3′ regions of *dtpT*, respectively, that were used for homologous recombination. Phenotypes of the relevant clones are shown on the *right*. *B*, Southern hybridization analysis of *Hin*dIII-digested chromosomal DNA of *L. lactis* strain MG1363 (*lane 1*), MG1363–3 (*lane 2*), and AG300 (*lane 3*). Labeled chromosomal fragment A was used as probe. Sizes (in kilobases) are indicated.

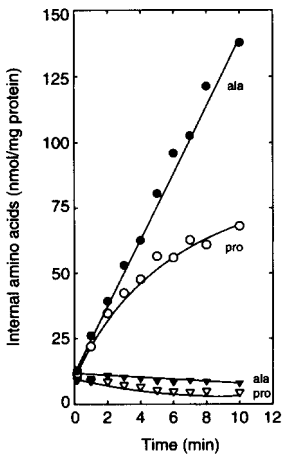

FIG. 8. **Time course of the internal alanine and proline pools in glucose-metabolizing cells of *L. lactis* MG1363 (○, ●) and *L. lactis* AG300 (▼, ▽) in response to addition of prolylalanine.** Prior to the transport assays cells were de-energized as described under "Materials and Methods." The cells were pre-energized for 2 min with 25 mM glucose in 100 mM potassium phosphate, pH 6.5, before the uptake assay was started by the addition of prolylalanine at 0.5 mM. Proline (▽, ○) and alanine (●, ▼) pools were determined as described under "Materials and Methods."

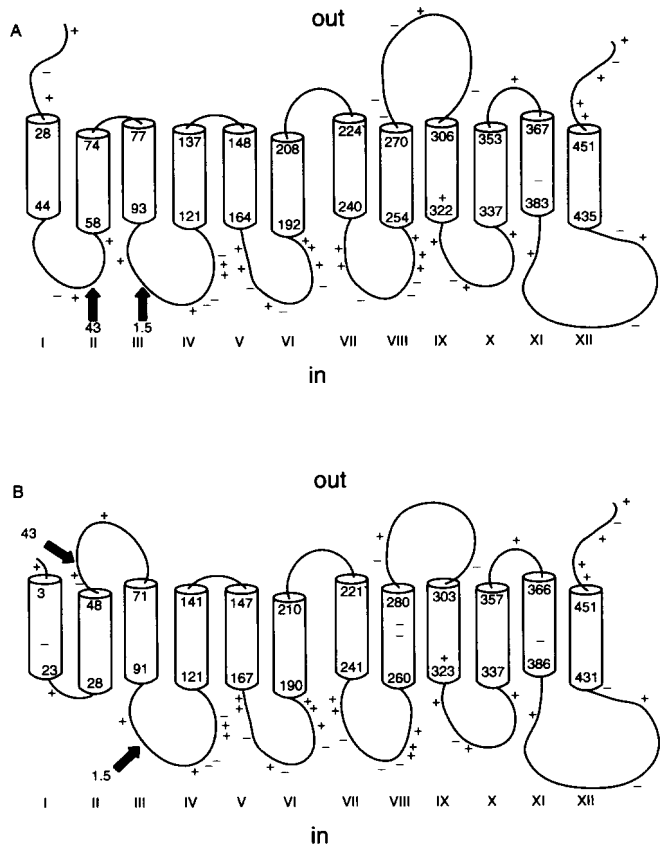

FIG. 9. **Topological models of the di-tripeptide transport protein of *L. lactis*, based on the "positive inside rule" (Von Heyne and Gavel, 1988), and the hydropothy profiling methods of Klein *et al.* (1985) (*A*) and Eisenberg *et al.* (1984) (*B*).** For the distribution of positive charges, histidine residues were not taken into account. The *arrows* mark the position of the DtpT-PhoA fusions. For the fusions at residue Asp-50 and Val-104 of DtpT, the "reverse" oligonucleotide primers 5'-CCCAACAACCCGGGAGCAACCC and 5'-ATAGATGC-CCGGGCATGTTAGA were used, respectively. The forward primer in the PCR reaction corresponded with 5'-GTTTGTTATGGATC-CTCTTTTTC (bp. 79–101 in Fig. 3). *Numbers* at the *arrows* indicate alkaline phosphatase activity of the corresponding DtpT-PhoA fusion proteins in Miller units.

ing in the deletion mutant *L. lactis* AG300, and that complementation occurs with the *dtpT* gene *in trans*.

Sequence Comparison and Membrane Topology—The amino acid sequence of DtpT has been compared with those of proteins in the EMBL SWISSPROT sequence data base. No striking similarity with any other protein in the data base was found, indicating that *dtpT* encodes a new type of bacterial peptide transport protein. Hydropathy analysis of DtpT reveals 12 hydrophobic stretches of at least 20 residues (Fig. 3, *underlined*) and these regions most likely span the membrane in α-helical configuration (Poolman and Konings, 1993). The transmembrane segments III-XII are indicated by different hydropathy analysis methods but differences are predicted for the amino-terminal portion of the protein (see Fig. 9, two alternative models). For both models, the "positive-inside-rule" (Von Heyne and Gavel, 1988) predicts that the amino- and carboxyl-terminal ends of the DtpT protein are located at the outer surface of the membrane. It should be noted, however, that the proposed interhelix loop XI-XII (about 44 amino acids) is long enough to span the membrane in α-helical configuration which would then bring the carboxyl terminus, with a surplus of two positive charges, at the inner surface of the cytoplasmic membrane. To discriminate between the two topology models (Fig. 9, *A* and *B*) and to obtain preliminary information about the location of the amino terminus, a number of in frame fusions of the 5′ region of *dtpT* and *phoA* were made. The positions of the fusion points and the corresponding alkaline phosphatase activities are shown in Fig. 9. The results support the model depicted in Fig. 9*B*, *i.e.* high alkaline phosphatase activity (43 Miller units) is observed with the fusion at residue Asp-50 of DtpT, which locates the corresponding loop region to the outside of the cytoplasmic membrane. The fusion at residue Val-104 yields low alkaline phosphatase activity (1.5 Miller units), indicating that interhelix loop III-IV is on the cytoplasmic side of the membrane.

DISCUSSION

Lactococci are multiple amino acid auxotrophs that require an exogenous nitrogen source for growth. They can use amino acids, peptides, and/or caseins to satisfy this requirement (Law, 1978). Di-tripeptide transport was shown to be essential for growth on the milk protein β-casein, indicating that one or more essential or growth-stimulating amino acids are released as di- or tripeptides during casein hydrolysis (Smid *et al.*, 1989b).

In this paper, the cloning of the di-tripeptide transport gene (*dtpT*) of *L. lactis* is described. The gene encodes a protein which shares no homology to components of any other bacterial peptide transport system nor with any other protein in the EMBL SWISSPROT sequence data base to date. The catalytic activity of DtpT also differs from other known bacterial peptide transport systems since it transports a wide variety of di-tripeptides in symport with proton(s).

The following observations indicate that the di-tripeptide transport gene of *L. lactis* was cloned. (i) Alanylglutamate uptake by dipeptide transport deficient *E. coli* cells harboring pDT5 is significantly higher than in cells which contain the vector pTAQI only (Fig. 5*A*). (ii) Substrate specificity studies indicate that DtpT specifically transports di- (and tri) peptides. (iii) A dipeptide transport-negative mutant, designated *L. lactis* AG300, has been constructed by homologous recombination using flanking regions of the cloned *dtpT* gene. (iv) Transformation of *L. lactis* AG300 with pDT5 resulted in growth characteristics similar to those of wild-type MG1363.

A few observations regarding peptide transport activities in

whole cells require further explanation. In our analysis of transport activities using intact cells the peptides are in most cases hydrolyzed rapidly in the cytoplasm. Since the amino acids formed can be metabolized and/or excreted at different rates, the estimated rates of peptide uptake will always be an underestimate and the observed accumulation of amino acids will be different for the various residues present in the peptide (see Fig. 8). A surprise has been the observation that *E. coli* E1772 is unable to hydrolyze Ala-Glu, which has allowed us to detect peptide exchange in whole cells. Finally, in our transport assays using washed cells of *L. lactis* or *E. coli* and the peptides indicated in this study, we have never detected any dipeptide hydrolysis outside the cell membrane.

Known bacterial peptide transport systems other than DtpT are encoded by an operon of (in many cases) five genes, and these systems all belong to the superfamily of ABC transporters. The oligopeptide transport system of *L. lactis* which transports peptides of 4 up to at least 6 amino acid residues (Kunji *et al.*, 1993), also belongs to this family (Tynkkynen *et al.*, 1993). In contrast to the oligopeptide transport system, the di- and tripeptide transport system of *L. lactis* is encoded by a single gene (*dtpT*). On basis of the longest ORF found, DtpT consists of 463 amino acid residues. The topology studies, *i.e.* hydropathy profiling and PhoA-fusion analysis, indicate that DtpT is most likely composed of 12 α-helical transmembrane domains and that the amino- and carboxyl-terminal ends face the outside of the cell membrane. Consistent with the proposed structure model is the absence of a relatively large cytoplasmic loop that separates the helices I-VI and VII-XII of (most) secondary transport proteins which have the amino and carboxyl termini at the cytoplasmic side of the membrane (Poolman and Konings, 1993). More detailed studies, however, are needed to substantiate the topological model presented in Fig. 9*B*.

Transport of di- and tripeptides by *L. lactis* is coupled to the proton motive force. It has been shown that Ala-Glu uptake is an electrogenic process and that the dipeptide is transported in symport with at least two protons (Smid *et al.*, 1989a). DtpT not only catalyzes the transport of Ala-Glu, but also that of neutral peptides like Ala-Ala, Leu-Leu, and others, and peptides with two negative charges like Glu-Glu and Asp-Glu. The driving force for transport of these peptides is unknown. Depending on the charge of the peptides and the number of protons that are cotransported, the actual driving force may vary for each type of solute. The construction of a genetically well-defined host strain (this study) and the overexpression of DtpT using plasmid constructs will allow us to answer these questions. Spontaneous diACA-resistant mutants were described earlier (Smid *et al.*, 1989b), but these mutants still possessed residual transport activity for some di- and tripeptides.

So far, proton-linked peptide transport systems have not been described for bacteria other than *L. lactis*. Proton-dependent dipeptide transport systems present in renal and intestinal brush border membranes of chicken, rat, rabbit, hamster, pig, and human have been described (Matthews and Payne, 1980; Berteloot *et al.*, 1981; Wilson *et al.*, 1989; Ganapathy *et al.*, 1981; Daniel *et al.*, 1992). These carriers transport di- and tripeptides that are resistant to hydrolysis by enzymes present in stomach and pancreas. Orally active α-amino β-lactams, which possess certain structural features of peptides, including a peptide bond with an α-amino group and a free carboxylic acid group, also use the uptake system for di- and tripeptides in brush border membrane vesicles (Okano *et al.*, 1986; Kramer, 1987). Putative protein components of the intestinal systems for uptake of peptides and β-lactam antibiotics have been identified using photoreactive substrate analogs. In enterocytes from rabbit, pig, and rat small intestine, an integral membrane protein of apparent M_r 127,000 was specifically labeled by these derivatives (Kramer *et al.*, 1988, 1990, 1992). No information about the genetics of these eukaryotic transport systems is available.

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